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Steam Sterilization of Apple Boxes For Blue Mold

by

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[1] In cooperation with the State Committee on the Relation of Electricity to Agriculture.
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STEAM STERILIZATION OF APPLE BOXES FOR BLUE MOLD

Richard H. Wellman and F. D. Heald

INTRODUCTION

Picking boxes that have been used for one or more seasons are recognized as a source of *Penicillium expansum* spores. Heald and Ruehle (7) recommended the use of clean lugs or picking boxes rather than old boxes loaded with spores, or the sterilization of old boxes if they must be used. Later Baker and Heald (2) stated that contamination of picking boxes by *P. expansum* spores increased from year to year and that only by disinfection could the number of viable spores be kept at a low level. They suggested the use of sprays of sodium hypochlorite having about .25 per cent available chlorine for effective disinfection of picking boxes and packing equipment

However, steam is available in most packing houses where it is the common source of heat used to warm the washing solutions. With this convenient source of supply, steam sterilization would seem to fit well into commercial practice provided that the time necessary for such sterilization is relatively short.

REVIEW OF LITERATURE ON HEAT TREATMENTS OF MOLDS

Hot water has been used for mold destruction by several workers and their recommendations vary as do the temperatures used and the materials on or in which the mold spores were found. Wallace and Tanner (22) and Lewis and Yesair (10) found that, although species of molds vary slightly in their thermal death times, a temperature of 60° C. for 5 minutes is usually sufficient to assure complete destruction. Lewis and Yesair (10) also found that *P. expansum* was destroyed in 15 minutes at 55° C. Streider and McClellan (19) submerged flasks containing mold suspensions in a water bath at 20° C. The temperature was slowly raised to 100° C. so that the elapsed time was 35 minutes. No mold survived.

Working in the presence of organic material (frankfurters) Roderick[1] found that all molds were killed when they were heated in water at 60° C. for 10 minutes. The lengthening of time of exposure over that given by Wallace and Tanner (22) may be readily explained by the presence of organic matter which has a protective effect on the spores. Macey and Pukrabet (13) recommended a 5-minute exposure to boiling water as a

[1] Quoted from Lewis and Yesair.

satisfactory mold sterilization of parchment paper. This recommendation may also be explained on the basis of the protective effect of organic matter. However, Hood and White (8) have found very short exposures (they do not state exact time), to boiling water to be effective for mold prevention in butter.

In churn sanitation, which Macy and his co-workers (12) have shown to be very important in the reduction of molds in butter, longer exposures to higher temperatures have been necessary. Macy (11) and Grimes, et al (6) attributed this to the fact that the wooden churns contained mold spores deep in the cracks where heat penetrated slowly. Here, as James (9) suggested ,the water probably did not come in actual contact with the contamination. Bendixen (3) reported that the treatment of the churn with ample quantities of water from 82-93° C. for at least 15 minutes was a satisfactory means of sterilization. Brown and Bouska (5) stated that water at the lowest temperatures (82° C.) recommended by Bendixen (3) was satisfactory for mold disinfection of open surfaces if exposures of 10-minute duration were used; though they suggested increasing the time of exposure if cracks were present in the surface. Morrison, Macy and Coombs (15) stated that an exposure of 30 minutes to water at 96° C. was necessary for surface disinfection; Neill (16) found no reduction in mold growth from wooden blocks held at 99° C. for 10 minutes; and James (9) stated that viable molds were worked from a churn after exposure to boiling water.

The pasteurization process should be thought of as closely allied to hot water treatments. The difference is that the water in the pasteurization process comes directly from the substance contaminated with the molds. In this connection it may well be brought out that high temperature, short-time (171° C. -15 sec.) pasteurization of milk has been found about as satisfactory as ordinary (62° C. -30 min.) pasteurization (14). Bouska's (4) work, in which he stated that spores of practically all molds were killed by a 30-second pasteurization at 79° C., might indicate considerable heat resistance in some mold spores, but spores of *Penicillium expansum* were found by Thom and Avery (21) to be susceptible to pasteurization treatments at lower temperatures than was necessary for a general recommendation. They stated that *P. expansum* was killed by pasteurization at 54.5° C. for 30 minutes, or at 68.3° C. for 30 seconds.

In butter pasteurization, as in hot water treatments of contaminated organic material, workers have found more severe pasteurization methods necessary. Milk apparently does not afford mold spores the protection that butter does. Hood and White (8) found that an exposure at 82° C. for 10 minutes gave a satisfactory means of mold prevention. Grimes, Kennelly, and Cummins (6) found that no molds in butter survived after the temperature had been raised to 85-90° C.

Streaming steam as an agent for mold destruction has been reported on by fewer workers but where used has given good results. Though exposures of 180 minutes to steam in churn sanitation were recommended by Macy, Coombs and Morrison (12), they did not report trying shorter exposures. A much shorter exposure (4 minutes) was used by Streider and McClellan (19) in their work on bread molds. They reported that Aspergillus species alone survived this treatment, in which bread contaminated with many molds including Penicillium sp. was exposed to the steam. Very short (time not stated) exposures to steam have been recommended by Hood and White (8) for mold prevention in butter.

Steam under pressure was also investigated by Streider and McClellan (19). They substituted a 30-second exposure to 15 lbs. steam pressure (110° C.) for a 4-minute exposure to streaming steam in the experiment previously given. After this treatment both Rhizopus and Aspergillus sp. survived.

Dry heat was found ineffective by these workers (19) in an experiment in which contaminated loaves were exposed to dry heat at 120° C. for 10 minutes. Both Aspergillus and Penicillium species withstood this exposure, although most of the spores were destroyed

As the general recommendation for complete sterilization is 20 minutes exposure to steam at 15 lbs. pressure, it would seem that organisms of the mold group are relatively susceptible to heat sterilization, and among the mold organisms it appears that *P. expansum* is one of those most susceptible to heat treatment. Different techniques may account for some of the varied results of the workers cited; however, the use of different organisms probably is responsible for a large portion of these discrepancies.

The problem of churn sanitation differs from that of apple box sanitation in that the contents of the churn are colloidal and worked into the walls of the churn. Thus, the churn becomes a suitable medium for mold growth and sporulation is produced, increasing the amount of inoculum. On the other hand, the apple box remains dry and the spores which are deep in the cracks are not in a position to contaminate the apples. Since this is the case complete sterilization of the cracks should not be so necessary in apple boxes as it is in churns.

METHODS USED

With these results in mind, work was undertaken to determine the effect of streaming steam upon the spores of *Penicillium expansum*. In an attempt to simulate field conditions small blocks of apple-box wood, approximately one-inch square, were used as vehicles of the blue mold spores. A suitable number of these spores were placed on the blocks by a method described later and then exposed to streaming steam for varying periods of time. After the attempt at sterilization had been made the spores were transferred from the wood to a 100 cc. water blank. One

cubic centimeter of this suspension was plated out in triplicate on 2 per cent potato-dextrose agar in petri dishes. After a suitable length of time the colonies of Penicillium that formed on the agar were counted and the total used as an index of the relative efficiency of the treatment.

In order that the number of colonies growing on plates poured from the series with no steam sterilization might be within the range that could be easily counted, an adapted form of the method of Baker and Heald (1) and of Wellman and Heald[1] was used. A portion of a 6-8 day old culture of *P. expansum* was removed by means of a sterile scalpel and placed in a 100 cc. water blank, and then vigorously agitated in order to separate the clumps of spores. A one cc. pipette (graduated in tenths) was used in transferring portions of this suspension to the blocks of apple-box wood previously mentioned. Preliminary experiments showed that in the above procedure one-half of a square centimeter of densely-sporulating colony of Penicillium used in making the spore suspension and one-tenth of a cubic centimeter of this spore suspension placed on the block, when plated out resulted in the formation of colonies, which were scattered enough to be easily counted and yet were frequent enough to lend numerical weight to the results. After the addition of the spore suspension to the blocks they were allowed to dry for approximately an hour and then were subjected to their respective sterilization treatments.

The other step in the procedure that necessitated the development of special technique was the removal of the spores from the wooden block. At first this was attempted by shaking the block in 100 cc. of sterile water for one minute. When a wooden block that had been treated in this manner was placed in a petri dish and covered with agar, innumerable colonies of Penicillium resulted. This inefficient method was discarded and, instead, each block was successively scraped with a sterile scalpel in a series of four washes of sterile distilled water in petri dishes using a total of 100 cc. This water was then transferred to its original Erlenmeyer flask and thoroughly shaken. The plating out was then conducted as first described. Table 1 shows the relative effectiveness of the two methods of spore removal and also shows the relative difficulties of removing the spores from the planed and unplaned surfaces of the blocks.

The results obtained in Table 1 indicate that it was relatively less difficult to remove spores from the unplaned than the planed surface of the block. In commercial usage the unplaned side of the box, being on the inside, comes into contact with the apples much more frequently than does the planed side Thus, the primary interest is in sterilization of the unplaned surface.

The method in which scraping with a scalpel in a series of washes was used, gives favorable results as is shown in Table 1. As a result of

[1] Unpublished experiments on chemical treatments of spores of *P. expansum*.

Table 1. Relative Efficiency of Methods of Spore Removal and Relative Difficulty of Spore Removal from Planed and Unplaned Surfaces of Blocks

Surface of block contaminated	Method of treatment of blocks	Colony count*				Total spores in suspension	Colonies from block**
		a	b	c	Av.		
Unplaned	Shaking in Erlenmeyer flask	4	2	8	5	500	Over 500
Planed	Shaking in Erlenmeyer flask	2	1	5	3	300	Over 500
Unplaned	4 washes with scraping	64	93	84	80	8000	42
Planed	4 washes with scraping	16	31	34	27	2700	94

* Colony count, gives the number of colonies in each of the three plates poured in a test.

** Colonies from block, shows the number of colonies formed on agar poured over the block after the test.

these preliminary trials the technique of scraping with a scalpel was followed in the sterilization tests.

EXPERIMENTS AND RESULTS

With the procedure thus established, the next step was a test with streaming steam to determine if this agent had any commercial promise. The blocks carrying the spores were placed in the laboratory autoclave; the door was tightly closed and the outlet valve fully opened. The inlet valve was partially opened for the required length of exposure, then quickly closed. The door was opened immediately and the blocks removed. The treated blocks were then scraped to remove the blue mold spores and the spore suspension resulting was plated out as has already been described in detail. The colonies were counted after an incubation period of 72 hours at laboratory temperature. The results of this experiment are recorded in Table 2.

Table 2. The Effectiveness of Streaming Steam as an Apple-box Sterilizer

Length of exposure in minutes	Colony count				Block count
	a	b	c	Av.	
None (check)	64	93	84	80	42
1	0	0	0	0	1
2	0	0	0	0	0
4	0	0	0	0	0
8	0	0	0	0	1
16	0	0	0	0	2

The colonies appearing on the agar poured over the blocks were probably of a species of Penicillium more resistant to streaming steam than *P. expansum.*

The results shown in Table 2 indicate that one-minute exposure to streaming steam under the conditions of the experiment was sufficient to kill all the spores of *P. expansum* present. These results were contrary

to the older recommendations to attain complete sterilization, which specified one to two hours' exposure to streaming steam. They agreed in general, however, with the experiments of the investigators cited at the beginning of this article, if temperature differences and absence of any protective organic matter in the experiment with blue mold spores are taken into account. With the older recommendations in mind the possibility of a flaw in technique was raised, consequently another experiment was conducted under comparable conditions. One possibility of misinterpretation of results was that an inhibition of colony formation rather than an actual killing of the spores was initiated by the steam exposures. This is known to be an actuality in sterilization with sodium orthophenylphenate[1], hence in the experiment presented in Table 3 the colonies were not counted until 144 hours had elapsed instead of the 72-hour period previously used.

Table 3. Retest in Autoclave of Streaming Steam as a Sterilizing Agent on the Spores of *P. expansum*.

Length of exposure in minutes	Colony count			
	a	b	c	Av.
None (check)	4	6	8	6
1	0	0	0	0
2	0	0	0	0
4	0	0	0	0

Table 3 shows the accuracy of the previous experiment. The technique of using the streaming steam was now subjected to suspicion, since it is comparatively easy to build up a pressure in an autoclave even with the outlet valve open. In order to determine this point another experiment was made using the Arnold sterilizer as a source of steam, since a pressure cannot be built up in this apparatus because of its construction. In order to give as accurate results as possible the blocks were placed in the sterilizer in such a manner that the spores were on the opposite side of the block from the steam This technique increased the length of time that was necessary for satisfactory sterilization as will be shown by later experiments. The results appear in Table 4.

Table 4. Test in Arnold Sterilizer of Streaming Steam as a Sterilizing Agent on the Spores of *P. expansum*

Length of exposure in minutes	Colony count			
	a	b	c	Av.
None (check)	12	19	14	15
½	2	3	4	3
1	1	2	1	1⅓
2	0	0	1	⅓
4	1	1	0	⅔

[1] Wellman and Heald. Unpublished experiments on chemical treatments of spores of *P. expansum*.

In this experiment a marked reduction in the number of colonies was noted with the two shorter exposures, while the two longer exposures still showed a very few colonies that were probably contaminants. Microscopic observation of the young colonies from spores, that had been subjected to the longer steam exposures, showed them to differ from the typical young colonies of *P. expansum*. Typical week-old colonies of *P. expansum* are Pistachio Green (Ridgway XLI) (17) in color, the colony surface appears fuzzy with short, loose coremium-like aggregates of conidiophores, the reverse side of the colonies is greenish-white in color, the conidia are smooth-walled, globose to slightly elliptical, 2.8 to 3.8 microns in diameter. The week-old colonies of the Penicillium thought to be a contaminant are a more yellowish green, the colony surface more velvety in appearance, with a slightly raised center portion, submerged hyphae in advance of sporulating area are lightly tinted yellow, the reverse side is yellowish green near colony edges and more deeply yellow towards the center, and the conidia are smooth-walled globose to sub-globose, 3.5 to 4.5 microns in diameter. This very nearly fits Thom's (20) description of the *P. viridicatum* series of which Ruehle (18) found three members to be weakly parasitic on apples.

In order to check the minimum exposure to streaming steam necessary to inactive spores of *P. expansum* in still another way, an experiment was conducted in which one series, used as a check, was exposed to steam in an Arnold sterilizer and another series exposed to steam at the mouth of a flask in which water was rapidly boiling. The technique for the Arnold sterilizer has been previously described. In using a flask as a steam source, a 2-liter flask was filled to the neck with water and brought to boiling over a radial gas burner. The block of wood was manipulated by sticking a scalpel firmly into the side opposite that on which the spores had been placed. The block was then lifted by the scalpel and the spores exposed directly to the steam at the mouth of the flask. This method allows the steam direct access to the spores and hence, may give better results than in the Arnold sterilizer method. The results of this experiment appear in Table 5.

Table 5. Comparison of Flask Method of Exposure to Streaming Steam with the Arnold Sterilizer Method

Source of steam	Length of exposure in minutes	Colony count			
		a	b	c	Av.
Check	None	9	8	5	7 ⅓
Flask	½	0	0	0	0
Flask	1	0	0	0	0
Flask	2	0	0	1	⅓
Arnold sterilizer	¼	3	0	0	1
Arnold sterilizer	½	3	3	0	2
Arnold sterilizer	1	1	0	0	⅓
Arnold sterilizer	2	0	1	1	⅔

The data recorded in Table 5 show that direct exposure to steam, as in the "flask method" is more effective than the indirect exposures used in the Arnold sterilizer. This experiment indicates that a direct exposure to streaming steam for ¼ minute is sufficient to kill all spores of *P. expansum* present.

If the contaminating colonies of Penicillium sp. came from the wooden blocks used, previous complete sterilization of these blocks would inactivate them and thus free the plates of these colonies. Therefore, the blocks were sterilized with steam at 15 lbs. pressure for 20 minutes, and then dried. The remaining procedure used was the same as in previous experiments.

Table 6. Comparison of Flask Method of Exposure to Streaming Steam with the Arnold Sterilizer Method, Using Previously Sterilized Blocks

Source of steam	Length of exposure in seconds	Colony count				Colonies from block
		a	b	c	Av.	
Check	None	32	32	36	33⅓	Over 500
Flask	15	0	0	0	0	0
Flask	30	0	0	1	⅓	0
Flask	60	0	0	0	0	0
Arnold sterilizer	15	38	50	38	42	Over 500
Arnold sterilizer	30	2	3	3	2⅔	2
Arnold sterilizer	60	1	0	0	⅓	1

The results showed that only one colony appeared in the one-minute exposure with the Arnold sterilizer series and that only one colony appeared on the plates for the entire "flask" series. These results indicate that spores of a Penicillium, differing from that of *P. expansum* were present on the blocks and were the source of contamination in the previous tests where unsterilized blocks were used.

The data in Table 6 confirmed the results presented in Table 5, showing that direct exposure to steam for a duration of 15 sec. is sufficient to kill a very high percentage of blue mold spores.

Another possible flaw in technique was that the spores on the block might not have been completely dried in the hour or two allowed. To test this possibility spore suspensions were placed on a series of blocks of apple-box wood. One group was allowed to dry for four hours and then exposed to streaming steam for different lengths of time; another group was allowed to dry for two weeks before exposure to streaming steam. Since Baker and Heald (2) have shown that there is no reduction in the number of viable spores of *P. expansum* after two months on wooden blocks, this point was not rechecked. The steam treatment was given in the Arnold sterilizer as has been perviously described. The results of this experiment appear in Table 7.

The data in Table 7 indicate that the more completely dried spores were more susceptible to the effect of streaming steam.

Table 7. Effect of Length of Drying of Spores on Subsequent Susceptibility to Killing by Streaming Steam

Time of drying on blocks	Length of exposure in seconds	Plate count a	b	c	Av.	Colonies from block
	Check (none)	32	32	36	33⅓	Over 500
4 hours	15	38	50	38	42	Over 500
	30	2	3	3	2⅔	2
	60	1	0	0	⅓	1
	15	0	0	0	0	0
2weeks	30	0	0	0	0	0
	60	0	0	0	0	0

The question now arose whether the presence of organic matter would tend to protect the spores against steam sterilization. In order to determine this point an experiment was conducted in which decayed apple tissue was mixed thoroughly with a great number of spores. This mixture was then spread on the wooden blocks with a small spatula. After the mixture of decayed apple tissue and spores had been thoroughly dried for approximately a week, the blocks were exposed to streaming steam in the Arnold sterilizer. The number of spores remaining viable after each treatment was determined by the use of plate cultures in triplicate as in previous cases. The results are shown in Table 8 with the inclusion of the analyses from Table 6 which records the results of similar exposures of spores without decayed apple tissue.

Table 8. The Protective Effect of Decayed Apple Tissue on Spores of *P. expansum* Against Streaming Steam

	Length of exposure in seconds	Colony count a	b	c	Av.	Colonies from block
Without decayed tissue	None (check)	32	32	36	33⅓	Over 500
	15	38	50	38	42	Over 500
	30	2	3	3	2⅔	2
	60	1	0	0	⅓	1
	None (check)		Over 500			Over 500
With decayed tissue	15		Over 500			Over 500
	30		Over 500			Over 500
	60		Over 500			Over 500
	120	0	0	0	0	0
	240	0	0	0	0	0

From the results in Table 8 it will be seen that the blue mold spores when mingled with decayed apple tissue are more resistant to steam sterilization than when exposed alone.

In view of the difficulty of sterilization of cracks and joints in churns it was decided to carry out an experiment determining the efficacy of steam as a sterilizer in the joints of the apple box. Wooden blocks, previously described, were soaked in a suspension of *P. expansum* spores, then removed and allowed to dry. The blocks were then nailed together in groups of two in order to simulate apple box joints. Since these blocks were about an inch square, spores in the center of adjoining surfaces were approximately ½ inch from the edge and from direct exposure to steam. This distance from direct exposure is greater than any found in normal apple boxes. These blocks were then subjected to streaming steam in the Arnold sterilizer as previously described. Tables 9 and 10 give results of two different experiments using this procedure.

Table 9. Efficacy of Streaming Steam in Joint Sterilization*

Length of exposure in minutes	Colony count				Colonies from block
	a	b	c	Av.	
Check	708	687	720	705	Colonies present over 500
½	191	162	140	164	Colonies present over 500
1	374	308	301	328	Colonies present over 500
2	0	0	0	0	1
4	0	0	0	0	0
8	0	0	0	0	0

*The smaller number of colonies found in the plates from blocks exposed 30 sec. than from those exposed 1 min. was due to the smaller area of the block exposed 30 sec.

Table 10. Efficacy of Streaming Steam in Joint Sterilization

Length of exposure in minutes	Colony count				Colonies from block
	a	b	c	Av.	
Check	280	418	357	352	Colonies present over 500
½	50	69	87	69	Colonies present over 500
1	72	51	102	75	Colonies present over 500
2	0	0	1	0	0
4	0	0	0	0	0
8	0	0	0	0	0

The variability in spore concentration on the different blocks as compared to the greater uniformity previously obtained was due to the immersion of the blocks in a spore suspension. This method offers more chance of variability than does the method in which measured amounts of inoculum are placed on the block because of variations of exposure and of surface area of the blocks.

Tables 9 and 10 show that Penicillium spores found in joints are protected from steam action but that this protection is not so great as that recorded by other workers for the more tightly compressed joints

in a churn. In view of the results obtained an exposure of 2 minutes to streaming steam should give a degree of sterilization consistent with commercial practice.

As these experiments had all been conducted on artificially contaminated boxes it was deemed advisable to test the effect of steam on naturally contaminated apple-box wood. A box was selected at random from a used box-pile where it had been exposed to the air for several months. Blocks were made from this box as previously described, and these blocks were subjected to streaming steam in the Arnold sterilizer. The results obtained in this experiment appear in Table 11.

Table 11. Steam Sterilization of Naturally Contaminated Apple-box Wood

Length of exposure	Colony count				Block count
	a	b	c	Av.	
Check	3	2	6	4	Over 500
Check	8	11	8	9	Over 500
30 sec.	0	2	4	2	Over 500
30 sec.	0	1	1	1	Over 500
1 min	0	0	0	0	1
1 min.	0	0	0	0	0

As the box selected for this experiment was very rough it was difficult to remove the spores by the method used. The results given in Table 11 show, however, that *P. expansum* spores on apple boxes are no more resistant to steam exposures when the box is naturally contaminated than they are when it is artificially contaminated.

SUMMARY

1. A direct exposure to streaming steam for one minute is sufficient to kill a very high percentage of the spores of *Penicillium expansum*.

2. Spores that have been dried for two weeks are more readily killed by streaming steam than those which have been dried for a few hours only.

3. Spores mixed with decayed apple tissue in contact with apple-box wood are more difficult to kill with streaming steam than spores free from decayed tissue.

4. Spores between two pieces of wood, as in joints, are somewhat protected from the action of streaming steam but under the experimental conditions were killed within two minutes.

5. Spores of *P. expansum* on artificially contaminated and on naturally contaminated box-wood did not differ appreciably in their resistance to streaming steam.

6. In commercial practice a two-minute exposure to streaming steam of old picking boxes, which are to be used again, would be sufficient to kill all blue mold spores present.

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